Journal of Surgical Oncology 1998;69:239–248

Molecular Characterization of CXCR–4: A Potential Brain Tumor-Associated Gene

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Background and Objectives: We have previously reported the isolation of a G protein-coupled receptor, CXCR–4, that is overexpressed in glioblastoma multiforme tumor tissue (GMTT), as compared to normal brain tissue (NBT).

Methods: Gene-specific RT-PCR, Northern blotting, and in situ hybridization techniques were used to study its expression in a variety of normal tissues, tumor tissues, and cell lines, as well as during development. Antisense CXCR–4 was overexpressed in glioblastoma cells to study its effect on cell proliferation.

Results: Gene-specific RT-PCR analysis indicated that the CXCR–4 gene is overexpressed in several malignant glioma tissues, breast tumor tissues and cell lines. Northern blot analysis indicated that CXCR–4 is expressed at high levels in certain leukemias, uterine cancer, and Burkitt's lymphoma cell line. The occipital and temporal lobe showed high levels of CXCR–4 in normal human brain. The CXCR–4 gene was expressed in all organs in the early stages of development (days 8–10). In adult mouse, CXCR–4 is expressed only in brain, spinal cord, bone marrow, and pituitary gland. Antisense CXCR–4 overexpression in glioblastoma cells caused inhibition of cell proliferation and induction of cellular differentiation in vitro. This suggests that CXCR–4 expression may play an important role during embryonic development and also in the genesis of human gliomas.

Conclusions: On the basis of CXCR–4 expression data and antisense overexpression data, we conclude that CXCR–4 plays an important role in the tumorigenic properties of brain, breast, and other tumor types. On the basis of its unique expression during mouse development, we conclude that it may play an important role in the normal functioning of brain, spinal cord, and bone marrow during development.

J. Surg. Oncol. 1998;69:239–248.

KEY WORDS: **glioma; G protein; chemokine receptor; development; brain; brain tumors**

INTRODUCTION

The transformation of normal human brain cells into gliomas occurs as a result of the accumulation of a series of cellular and genetic changes, which include loss of

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Accepted 9 July 1998

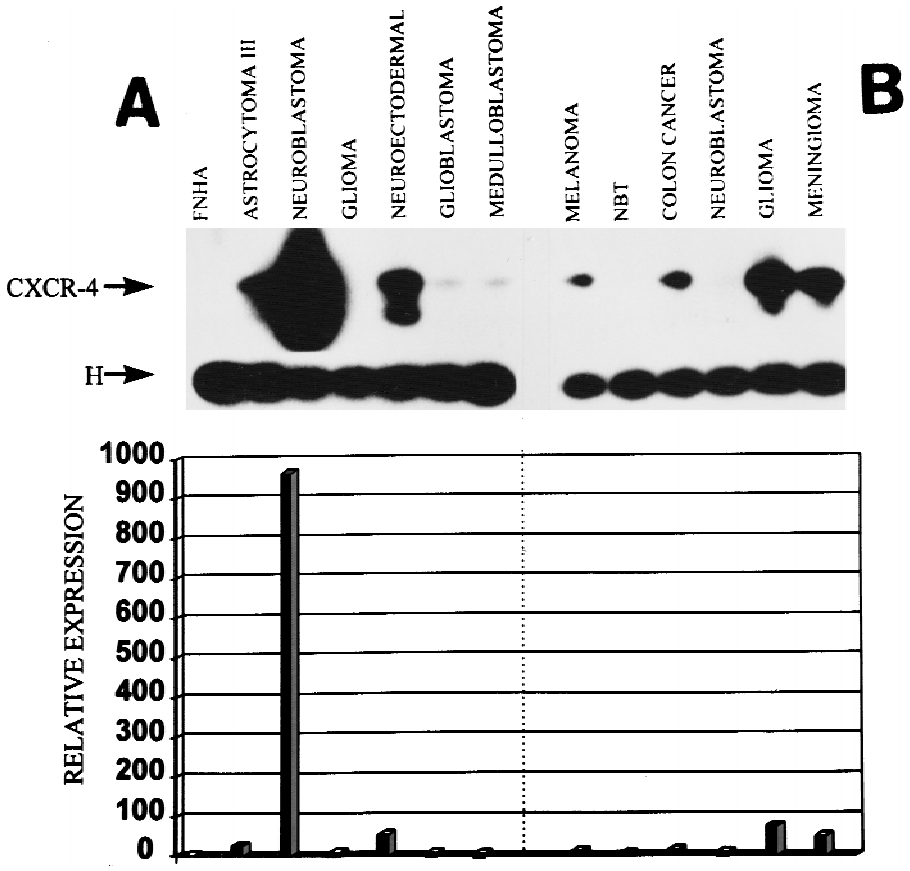

Fig. 1. Expression of CXCR–4 in human primary brain tissues **(A)** and cell lines **(B)**. Autoradiogram of a reverse transcriptase-polymerase chain reaction (RT-PCR) Southern blot of CXCR–4 expression *(top)* and D1–2 expression *(middle)*, represented by H, and that serve as an internal control for RT-PCR. Quantitation of Southern blots was performed using the ImageQuaNT™ volume quantitation program from the Molecular Dyamics Phosphor Imager. Relative expression of CXCR–4 after normalizing with D1–2 gene expression is illustrated as a histogram *(bottom).*

17p, 13q, 9p, 19, 10, 22q, and 18q and amplification of 7 and 12q chromosomes [1–3]. These alterations lead to change in the expression of several genes (*p53, RB, IFN*α/β, *CDKN2, MMAC1, DCC, EGFR, PDGF, PDGFr, MDM2, GLI, CDK4,* and *SAS*) during the genesis and progression of human gliomas [1,2]. Despite identification of these alterations, the exact series of events that lead to the genesis of human gliomas is unclear. All these genetic changes are observed in only a fraction of tumor samples. This is apparently attributable to the polyclonal nature of the tumor. Thus, there is an immediate need to identify more glioblastoma multiforme tumor-associated markers.

Using the technique of differential hybridization of the Atlas™ Human cDNA expression array technique, we identified that the CXCR–4 gene is overexpressed in glioblastoma multiforme tumor tissue (GMTT), as compared with normal brain tissue (NBT) [4]. CXCR–4 is a member of the seven–transmembrane G (guanosine-nucleotide-binding) protein-coupled receptor family of proteins. Four other G protein-coupled receptors are known to be linked with the process of neoplastic transformation and include (1) MAS oncogene (neuronal angiotensin-sensitive receptor); (2) serotonin 5HT1c receptor; (3) muscarinic acetylcholine receptor (mAChR), and (4) α_{1B}-adenergic receptor (α_{1B}-ADR) [5–8].

CXCR–4 was first identified as a cDNA that was amplified using degenerate primers made against leukocyte chemotactic factor receptors (N-formyl peptides, C5α and IL–8) and was termed HM89 [9]. The amino acid sequence of HM89 has 92% identity to a bovine clone LCR1 (locus coeruleus), a neuropeptide Y (NPY) receptor [9]. On the basis of strong sequence identity between HM89 and LCR1 it was initially speculated that HM89 is a human homologue of the bovine LCR1 clone, but ligand-binding analysis indicated that HM89 does not bind to NPY [9]. This clearly indicates that HM89 is not a NPY receptor, but its sequence features (such as seven transmembrane regions, glycosylation and phosphorylation sites) clearly indicate that it does belong to the G protein coupled receptor family. Amino acid sequence analysis indicates that HM89 has 36% identity to other leukocyte chemoattractants such as interleukin–8 (IL–8), angiotensin 2 type 1 receptor, but only 21% identity to

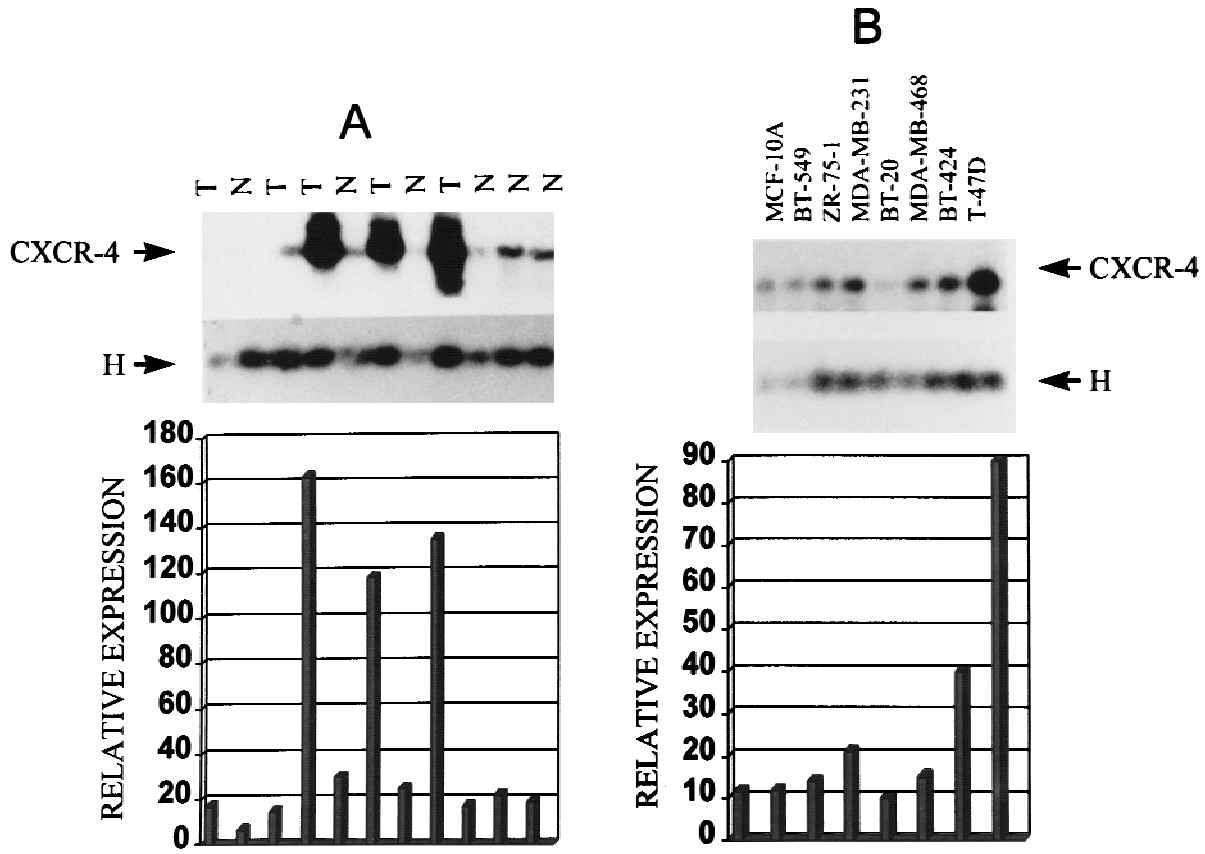

Fig. 2. Expression of CXCR–4 in primary breast tumor tissues **(A)** and cell lines **(B)**. Autoradiogram of a reverse transcriptase-polymerase chain reaction (RT-PCR) Southern blot of CXCR–4 expression *(top)*, and D1–2 expression *(middle)*, which serve as an internal control for RT-PCR. D1–2 gene is indicated by letter H. Relative expression of CXCR–4 after normalizing with D1–2 (indicated by letter H) gene expression is illustrated as a histogram *(bottom)*.

human NPY Y1 receptor [9]. HM89 does not have a significant homology to major members such as β_2-adrenergic receptor and rhodopsin, of the G (guanosine monophosphate) protein-coupled receptor family. Cytogenetic analysis indicates that HM89 is located on human chromosome 2q21[10]. In 1994, HM89 was re-cloned using a rabbit IL–8 receptor cDNA upon screening a human monocyte library and was named leukocyte-derived seven–transmembrane domain receptor (LESTR) [11]. In 1996, a cofactor for human immunodeficiency virus–1 (HIV–1) fusion and entry into the $CD4^+$ cells was isolated and sequenced [12]. This cofactor is identical to previously cloned HM89 and, because of its role as a fusion protein between the HIV–1 and $CD4^+$ cells, it was designated ''fusin.'' Sequence identity analysis indicated that HM89, LESTR and fusin are all the same gene. Because of its chemoattraction properties, HM89 is now termed CXC-chemokine receptor–4, CXCR4. Recently, it has been shown that CD4-independent infection by HIV is mediated by CXCR–4 [13,14]. The ligand for CXCR4 has recently been cloned and termed stromal cell-derived factor–1(SDF–1) [15]. We report in this paper that the CXCR–4 gene is overexpressed in a variety of brain and breast tumor tissues and cell lines. We also confirm the differential expression of CXCR–4 during development as previously reported by other workers [16]. Because CXCR–4 is overexpressed in a variety of tumor tissues and cells, it is likely that it may play an important role in the process of cell transformation and tumorigenesis.

MATERIALS AND METHODS

Human Tissues and Cell Lines

Brain and non-brain tumors and normal tissues were procured from the tissue bank maintained by Pacific Northwest Cancer Foundation, Northwest Hospital (Seattle, WA) and from the Mayo Clinic (Rochester, MN). Brain tumor glioblastoma cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Fetal normal human astrocytes (FNHA) were purchased from Clonetics (San Diego, CA). All the cell lines were cultured under the conditions recommended by the ATCC or Clonetics. Breast tumor cell lines were kindly provided by Dr. Rau Pan Huang, Ph.D. (Molecular Medicine, Pacific Northwest Cancer Foundation, Seattle, WA).

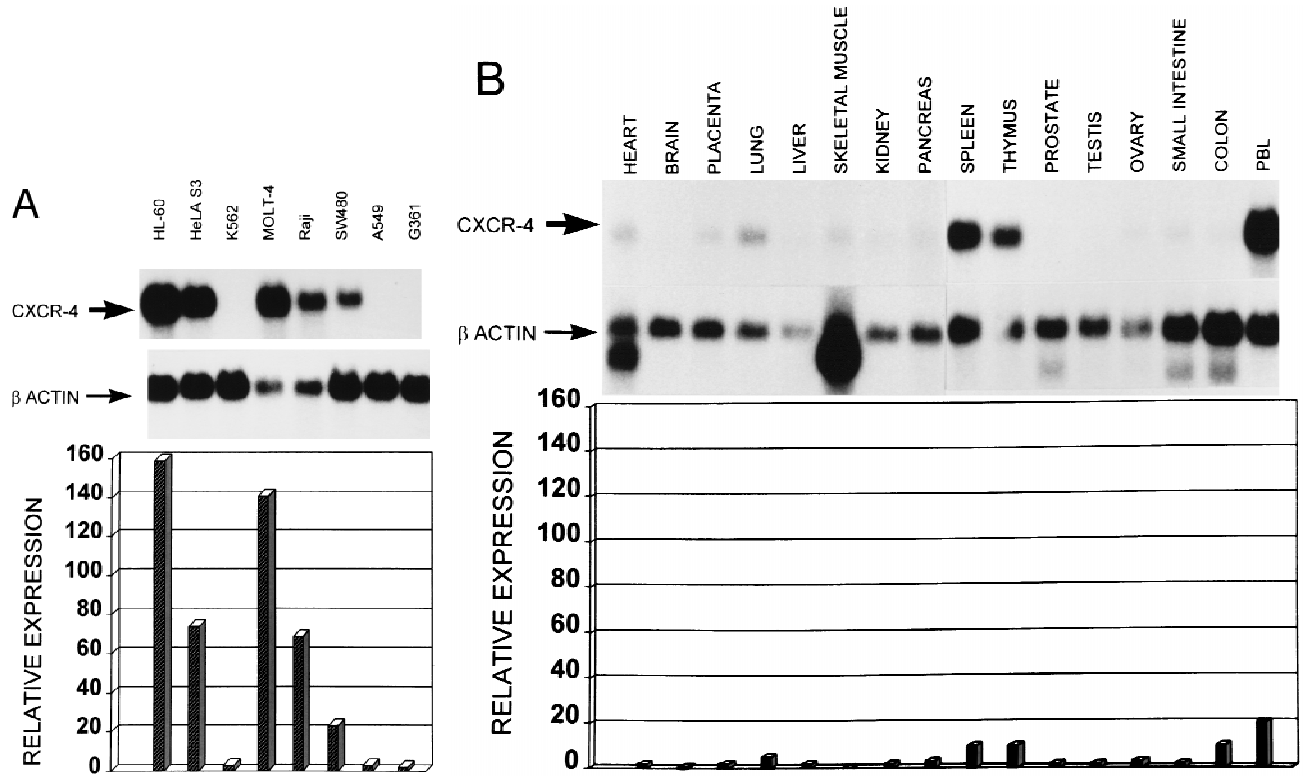

Fig. 3. Expression of CXCR–4 in human cancer cell lines **(A)** and in normal human tissues **(B)**. Relative expression of CXCR–4 after normalizing with β-actin expression is shown as a histogram *(bottom)*.

Gene-Specific RT-PCR

Gene-specific CXCR–4 primers (5′ctctccaaaggaaagcgaggtggacat3′,5′agactgtacactgtaggtgctgaaatca3′) were used for carrying out polymerase chain reaction (PCR). PCR for D1–2 (a mitochondrial cytochrome C oxidase subunit 1 gene, accession number D38112), was carried out using specific primers (5′cggagcaatatgaaatgatct3′, 5′gcaaatacagctcctattg3′). PCR was carried out as described in detail previously [17]. The PCR product was then run on a 1.2% agarose gel. DNA was transferred on to Hybond N^+ magnacharge membrane (Amersham, Arlington Heights, IL) using the standard Southern blot procedure as described previously [18]. Hybridization was carried out using 1×10^6 cpm/ml gene-pecific probe at 42°C for 18 h. Gene-pecific probes were prepared by multiprime labeling the internal primers (CXCR–4, 5′atctgtttccactgagtctgatcttcaagttttcacccagctaacaca3′ and housekeeping gene D1–2, 5′taggcctgactggcattgtattagcaaactcatcactaga3′) using the megaprime labeling kit from Amersham. The D1–2 gene has been used in the past as a housekeeping gene in the reverse transcriptase (RT)–PCR application [17]. Quantitation of RT-CR Southern blots was performed using the ImageQuaNT™ volume quantitation program from the Molecular Dyamics Phosphor Imager (Sunnyvale, CA). Volume quantitation calculates the volume under the surface created by a 3–D plot of pixel locations and pixel values. We quantitated the volume (the integrated intensity of all the pixels in the spot excluding the background) of CXCR–4 bands in Northern blots. These pixel values were then normalized with pixel values in the bands of the housekeeping genes (D1–1 or β-actin) and were indicated as relative expression.

Northern Blot Analysis

To study the expression of CXCR–4 in normal human tissues and in human cancer cell lines, two multiple normal human tissue blots (MNHTB) and a cancer human cell line blot (CHCB) were purchased from Clontech (Palo Alto, CA). These blots contained 2 μg of pure $polyA^+$ mRNA. Both normal and cancer blots were prehybridized in express hybridization buffer solution (Clontech) for 3–4 h. Hybridization was done with multiprime labeled 0.55-kb (positions 1591–1618) CXCR–4 probe. The CXCR–4 probe was then removed, and the human β-actin gene was used as an internal control.

Zoo Blot Analysis

A zoo blot membrane containing 5 μg of genomic DNA (digested with *Eco*RI restriction endonuclease enzyme) was purchased from Clontech. The zoo blot was prehybridized according to the method recommended by

Clontech. A 0.55-kb (1061–1618) CXCR–4 fragment was labeled with radioactive dATP^{32}(3,000 Ci/mmole) using decamer primer labeling kit from Ambion (Austin, TX) and was used as a probe for hybridization. Washing of the blot was performed as recommended by Clontech.

Cloning of the Full-Length CXCR–4 Gene

A human fetal brain library (Stratagene, La Jolla, CA) was screened with a CXCR–4 specific 0.55-kb PCR product (isolated from a neuroblastoma cell line using CXCR–4 specific PCR primers). Three positive clones were identified, and single plaques were isolated after secondary screening of the library. To assess the insert size for these clones, PCR was performed using *pfu* Taq DNA polymerase (Stratagene). PCR product was run on a 1.2% agarose gel. Sequence analysis indicated that clone No. 3 contained a 2.0–kb insert that was identical to the previously isolated full-length CXCR–4 clone. To clone CXCR–4 in the antisense direction, CXCR–4 specific primers (5′agatagatccgcgggtgtta gctggagtgaaaacttga3′ and 5′tagatacaactagtaccatggaggggatcagtatata3′) were used for carrying out the PCR and cloned into the predigested pCMVneo vector in sense and antisense direction. The orientation of CXCR–4 gene was confirmed by sequencing.

Cell Transfection and Proliferation Assay

5GB and HTB16 glioblastoma cells were purchased from ATCC. Cells were plated at an approximate density of 1×10^4 cells/cm^2. At 24 h after plating, the cells were washed with serum-free medium and transfected with lipofectamine reagent plus 5 μg plasmid DNA (pCMVneo or pCMVCXCRAS) diluted in 1 ml (final volume) of serum-free medium. Cells were incubated at 37°C for 5 h, after which the reagent was replaced with medium containing 10% fetal bovine serum (FBS). Cells were incubated at 37°C for 72 h. Medium was changed to the one containing 1,000 μg/ml G418. Cells were incubated at 37°C for 7 days. At this point, medium was changed to a single medium containing 200 μg/ml G418. Cultures were maintained at 200 μg/ml G418 indefinitely, changing medium every 72−96 h. pCMVneo or pCMVCXCRAS transfected 5GB and HTB16 glioblastoma cells are plated in triplicate at a density of 2×10^3cells/cm^2. Cells were harvested every 48 hand counted on a cell counter.

In Situ Hybridization

In situ hybridization was performed as described previously [19]. Briefly, 6 μm 4% paraformaldehyde fixed, paraffin-embedded mouse embryo sections from Novagen (Madison, WI) were deparaffinized by two washes in xylene, followed by rehydration through graded concentrations of ethanol from 100% to 70%. They were then washed in phosphate-buffered saline (PBS) and treated

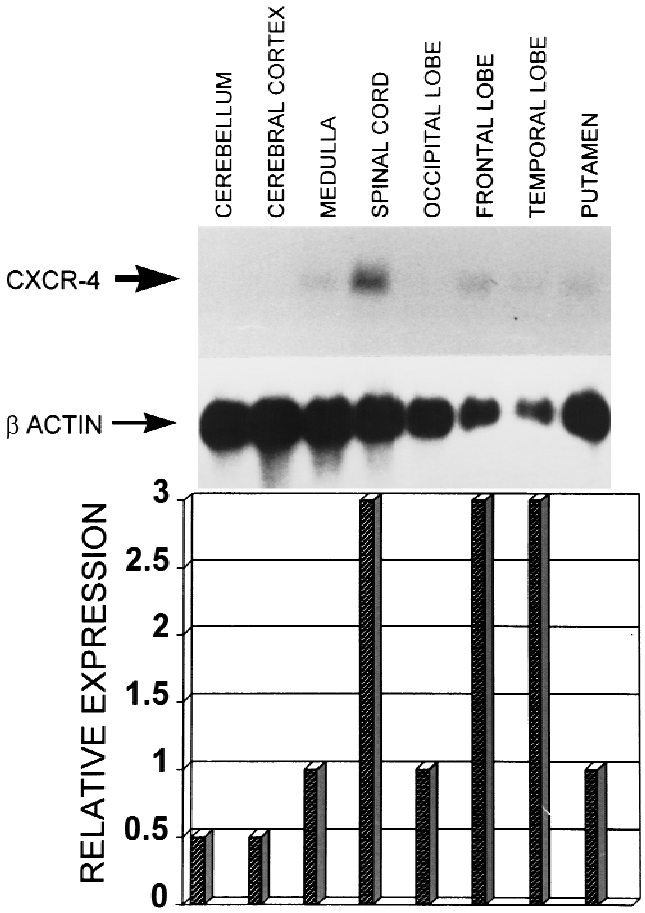

Fig. 4. Expression of CXCR–4 in different regions of the normal human brain. Autoradiogram of a multiple tissue Northern blot of CXCR–4 expression *(top)*, and β-actin expression *(middle)*, which served as an internal control for reverse transcriptase-polymerase chain reaction (RT-PCR). Relative expression of CXCR–4 after normalizing with β-actin expression is shown as a histogram *(bottom)*.

with proteinase K (25 μg/ml for 10 min), followed by fixation in 4% paraformaldehyde. After incubation in 0.25% acetic anhydride/0.1 M triethyl acetic acid (TEA), sections were dehydrated through graded concentrations of ethanol from 70% to 100% and prehybridized for 2 h at 55°C in 50% formamide, 5 × sodium chloride, sodium citrate (SSC) pH 4.5, 50 μg/ml tRNA, 50 μg/ml heparin, and 1% sodium dodecyl sulfate (SDS). Sections were hybridized with 1 μg/ml digoxigenin (DIG)-labeled antisense or sense probes for 18 h at 55°C. Probes were synthesized with the Genius 4 kit (Boehringer-Mannheim, Indianapolis, IN) using the T3 and T7 promoters of a PCR template derived from human CXCR–4 cDNA corresponding to bases 1061–1618 [10]. The PCR template was amplified using primers 5′ caagctcgaaattaaaaccctcactaaaggg ctctccaaaggaaagcgaggtggacat 3′ and 5′ cacttaactaatacgactcactatagggagactgtacactgtaggtgcgaaatca 3′ which contain the T3 and T7 promoters, respectively, added to the human CXCR–4 sequence corresponding to bases 1061–1087 and 1591–1618. After

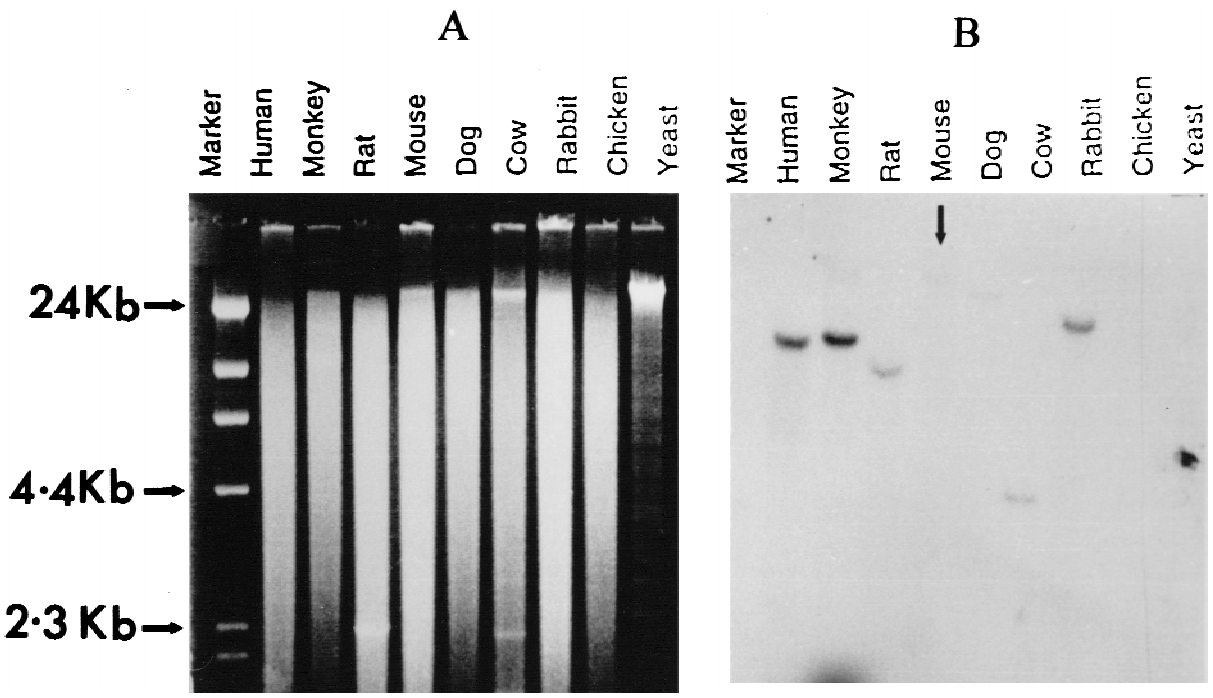

Fig. 5. Analysis of CXCR–4 sequence conservation in different animals. **A:** Ethidium bromide-stained gel of genomic DNA isolated from different animals. **B:** Autoradiogram of the genomic Southern blot hybridized with CXCR–4–specific probe. *Arrow*, CXCR–4 genomic DNA band in the mouse lane. Refer to Materials and Methods for details.

hybridization, these slides were washed in 50% formamide, 2 × SSC pH 4.5, 1% SDS at 50°C, treated with 5g/ml RNase A for 30 minutes at 37°C, and washed in 50% formamide, 2 × SSC pH 4.5 at 50°C. Sections were preblocked in 10% normal sheep serum (Sigma Chemical Co., St. Louis, MO) and incubated with a 1:2,000 dilution of alkaline phosphate conjugated anti-dioxigenin Fab fragments (Boehringer-Mannheim) for 18 h at 4°C. For detection, slides were incubated with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indilyl-phosphate (BCIP), 4-toluidine salt solution, in the dark for 28 h. After counterstaining with Eosin Y, slides were mounted with Permount and visualized using an Axioskop (Carl Zeiss, Thornwood, NY) routine microscope.

RESULTS

Expression of CXCR–4 in Glioblastoma and Other Brain Tumor Cell Lines and Tissues

We studied the expression of CXCR–4 in several brain tumor cell lines and primary brain tumor tissues using the RT-PCR technique. As shown in Figure 1A, CXCR–4 was expressed at high levels in neuroblastoma and neuroectodermal human tumor cell lines. Moderate levels of expression of CXCR–4 were observed in medulloblastoma and astrocytoma grade III cell lines (Fig. 5B). In primary tissues, high levels of CXCR–4 expression were observed in glioma and meningioma tumors (Fig. 1B).

Expression of CXCR–4 in Breast Tumor Primary Tissues and Cell Lines

We then studied the expression of CXCR–4 in 11 primary breast tissues (5 tumors and 6 normal). As shown in Figure 2A, CXCR–4 was expressed at high levels in three of the five breast tumor tissues studied and at low levels in six normal breast tissues. Two of the three breast tumor tissues that overexpress the CXCR–4 gene were estrogen and progesterone receptor positive, and one was negative for both receptors. We then studied the expression of CXCR–4 gene in one normal and seven breast tumor cell lines. As shown in Figure 2B, high levels of CXCR–4 expression were observed in only two cell lines, BT–424 and T–47D.

Expression of CXCR–4 in Cancer Cell Lines

The results above demonstrate that CXCR–4 was overexpressed in brain and breast tissues. In order to determine whether CXCR–4 gene is overexpressed in other tumor types, we studied its expression in a variety of cancer cell lines using Northern blot analysis. As shown in Figure 3A, high levels of CXCR–4 expression were observed in promyelocytic leukemia HL–60, HeLa cells S3, lymphoblastic leukemia, Burkitt's lymphoma Raji, and low levels in colorectal adrenocarcinoma SW 480. No expression of CXCR–4 was observed in lung carcinoma A549, melanoma G361, and chronic myelogenous leukemia K–562.

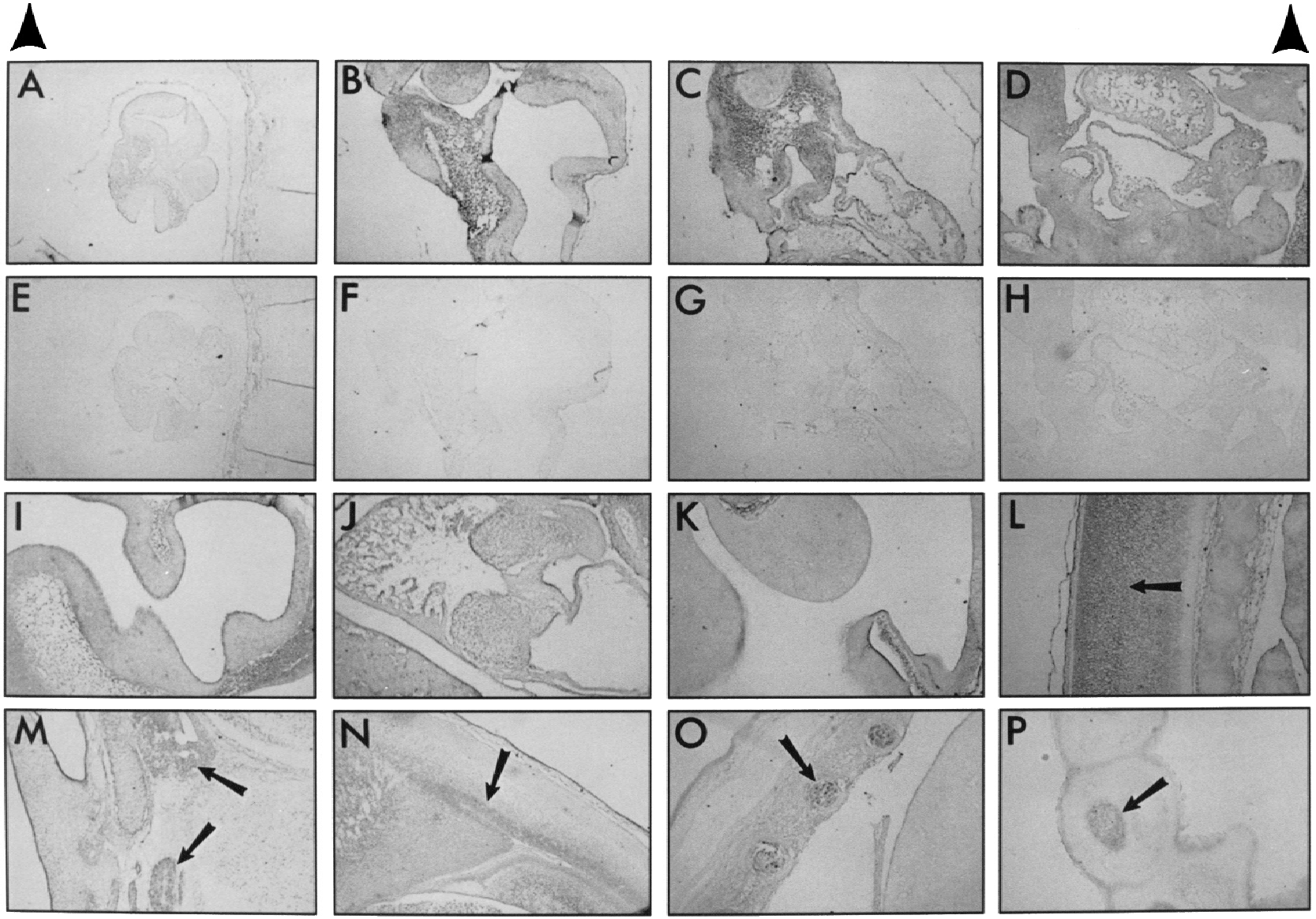

Fig. 6. Expression of CXCR–4 in mouse embryos during development. **A–D:** CXCR–4 expression in mouse embryos at 8 and 9 days (head region), 9 days (organ region), and 10 days (organ region), respectively, using antisense probe. **E–H:** Same as A–D, but instead hybridized with CXCR–4 sense probe. **I–O:** CXCR–4 expression in mouse embryos at 10 days (head region), 11 days (heart region), 11 days (forehead region), 13 days (spinal cord), 15 days (pituitary), 14 days (ribs, near spine), and 16 days (forelimbs), respectively, using antisense probe. *Arrows*, high level of CXCR–4 expression. *Thick arrows*, orientation.

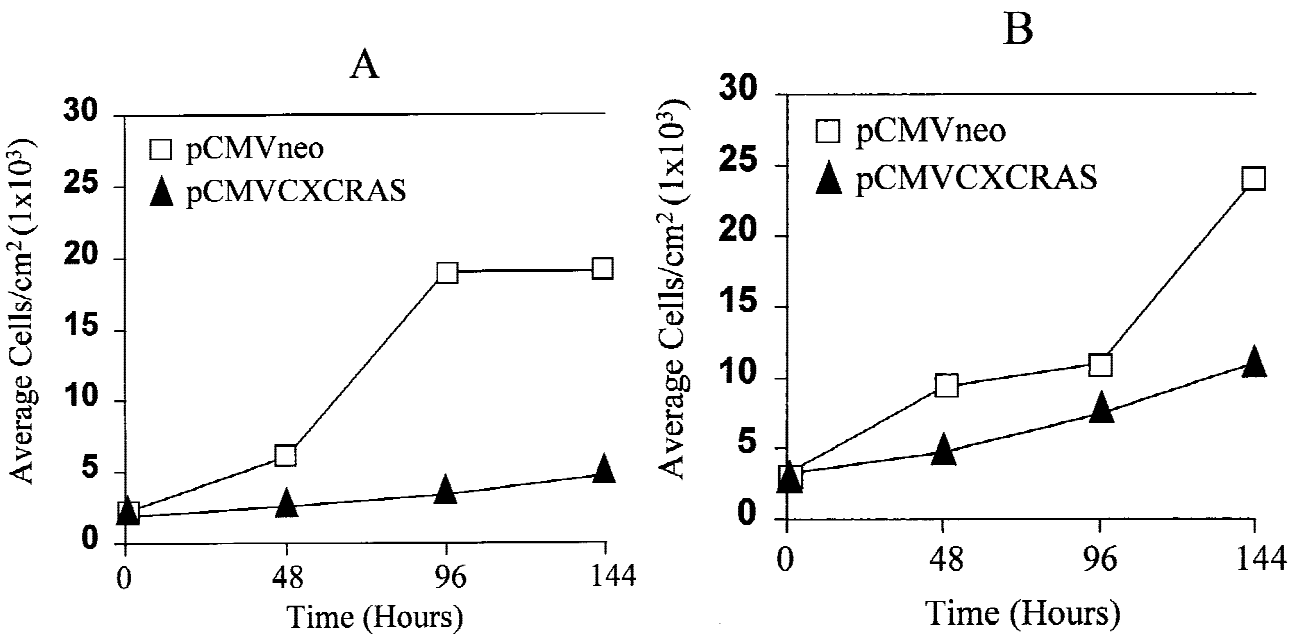

Fig. 7. Effect of CXCR–4 overexpression on the proliferation of human glioblastoma tumor cells. **A:** Effect of antisense CXCR–4 overexpression on 5GB glioblastoma cells. **B:** Effect of antisense CXCR–4 overexpression on HTB–16 glioblastoma cells.

Expression of CXCR–4 in Normal Human Tissues

To begin to understand the role of the CXCR–4 gene in normal cell function, we studied its expression in several normal human tissues using Northern blot analysis. As shown in Figure 3B, high levels of CXCR–4 was expressed in only four organs: spleen, thymus, colon, and peripheral blood leukocytes. Low expression was observed in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, prostate, testis, ovary, and small intestine.

Expression of CXCR–4 in Different Regions of the Human Brain

We used the technique of Northern blotting to study the expression of CXCR–4 in different regions of the human brain. As shown in Figure 4, the frontal lobe, temporal lobe, and spinal cord expressed higher level of CXCR–4. The functional significance of such selective expression in these three areas of the brain is unknown.

Expression of CXCR–4 During Mouse Development

Genes that are known to be upregulated during the process of tumorigenesis are also sometimes overexpressed during early stages of development [17]. It has been reported in the past that CXCR–4 is differentially expressed during rat embryonic development [16]. To confirm this observation, we have studied its expression during the early stages of mouse development. Before proceeding with the in situ hybridization of mouse embryos, we performed a zoo blot analysis to demonstrate that the sequence of CXCR–4 is conserved among human and mouse. As shown in Figure 5, CXCR–4 sequence is conserved among human, monkey, rat, mouse, dog, cow, and chicken.

Eight developmental stages of mouse embryos (days 8–16) were analyzed for CXCR–4 expression, using the technique of in situ hybridization. CXCR–4 expression was observed in all tissues from days 8–12. By day 14, high levels of expression were observed in most of the tissues and all regions of the brain and bone marrow. By day 15, expression was very strong in the forebrain and midbrain and in the pituitary gland. Low levels of expression were seen in the bone marrow, gut, and ovary. By day 16, CXCR–4 expression was mainly confined to brain and bone marrow. Some selective panels of CXCR–4 expression in different organs of mouse embryo are shown in Figure 6. This set of data confirms a previously published report that CXCR–4 is expressed in selected set of organs during development [16]. From this set of data, it is inferred that CXCR–4 most likely has some important function during the early stages of development.

Effect of CXCR–4 Overexpression in Glioblastoma Cell Lines

We demonstrated above that CXCR–4 is overexpressed in three glioblastoma cell lines analyzed. In order to assess the functional role of CXCR–4 in brain tumorigenesis under in vitro conditions, we first cloned a full-length cDNA of CXCR–4 from a human fetal brain library (see under Materials and Methods) and then subcloned into the pCMVneo vector in the anti-sense

direction for transfection into glioblastoma cell lines. We transfected 5GB and HTB16 cell lines with vector alone or in the antisense direction (pCMVneo, pCMVCX-CRAS). As shown in Figure 7, antisense CXCR–4 caused a reduction in the rate of glioblastoma cell proliferation. The effect of antisense CXCR–4 was much more dramatic in 5GB cells than in HTB16 cells.

DISCUSSION

Chemokines play an important role in the activation of immune cells both in vitro and in vivo. Chemokines produce their effect on cells by interacting with specific receptors on the cell surface. Several chemokine receptors have been isolated and cloned. A family of chemokine receptors that belong to C-X-C category have gained extensive attention because of their role in immune system regulation. These receptors can also interact with G proteins to send signals to the nucleus. Thus, they are also referred to as G protein-coupled receptors (GPRs). GPRs have been studied extensively for their role in normal cellular processes such as secretion and steroidogenesis. Several other GPRs (serotonin 5HT1c receptor, muscarinic acetylcholine receptor, and, α_{1B}-adrenergic receptor) have been studied for their role in cell transformation in vitro. One G protein-coupled receptor that has cell transforming ability in vitro and tumorigenic properties in vivo is the MAS oncogene. Serendipitously, we identified that CXCR–4, a chemokine receptor and GPR, is overexpressed in GMTT, as compared with NBT [4]. Our results clearly demonstrate that CXCR–4 is not only expressed in brain tumors, but also in breast tumors, neuroblastomas, leukemias and Burkitt's lymphomas. We found a high level of CXCR–4 expression in three of five breast tumor samples. To our knowledge, this is the first evidence of the presence of CXCR–4 in breast tumor samples. We also found overexpression of CXCR–4 gene in promyelocytic and lymphoblastic leukemic cell lines. It is unknown whether the overexpression could be the result of gene amplification. Experiments are under way to address this question.

Because of its important role as a HIV entry coreceptor, expression of CXCR–4 has been studied extensively by other workers [12,13]. The CXCR–4 gene product has been detected in differentiated human neurons, cultured endothelial cells, and in arterial endothelium [20,21]. The expression of CXCR–4 was recently studied in the rat during development [16]. Their results also demonstrated differential expression of CXCR–4 in thymus and in the proliferating areas of the brain.

What is the mechanism of CXCR–4-mediated cell transformation? Most cell surface receptors influence the cell function by sending signals to the nucleus. Upon activation with a ligand, the GPRs interact with a guanine nucleotide binding protein or G protein, which in turn causes the α-subunit to exchange a bound GDP for GTP. This leads to initiation of signal transduction to the nucleus [22]. The signal transduction of a typical GPR to the nucleus may involve a number of cytoplasmic components such as cAMP, PKA, c-Raf, MEK, MAPK, and AP–1. The C-terminus of GPRs contain phosphorylation sites that can influence their signal transduction to the nucleus. In a recent study, it was demonstrated that when the cytoplasmic tail of the CXCR–4 protein was removed, it resulted in higher G-protein activation, inositol phosphate generation and a more sustained calcium elevation [23]. SDF–1 and phorbol 12–myristate 13–acetate (PMA), but not a membrane-permeable cyclic adenosine monophosphate (cAMP) analogue, induced rapid phosphorylation as well as desensitization of the CXCR–4 receptor. In addition, it is speculated that signaling and internalization of CXCR–4 are regulated by receptor phosphorylation-dependent and -independent mechanisms. Analysis of the CXCR–4 protein indicated the presence of several protein modification and potential phosphorylation sites. Phosphorylation of these residues can alter the function of this protein. To understand the role of these protein modification sites, deletion mutations are currently being created.

On the basis of data presented in this paper, we conclude that CXCR–4 is a good marker for brain and breast tumors. Development of novel gene or immune based strategies to block the CXCR–4 expression or its receptor function will be very useful in the effective treatment of malignant human gliomas, and perhaps breast cancers.

REFERENCES

1. Furnari FB, Huang HJ, Cavenee WK: Genetics and malignant progression of human brain tumors. [Review.] Cancer Surv 1995; 25:233–275.
2. Sehgal A: Molecular changes during the genesis of human gliomas. [Review.] Semin Surg Oncol 1998;14:3–12.
3. Shapiro WR, Shapiro JR: Biology and treatment of malignant glioma. [Review.] Oncology 1998;12:233–246.
4. Sehgal A, Boynton AL, Young RF, et al.: Application of the differential hybridization of Atlas™ human expression arrays technique in the identification of differentially expressed genes in human glioblastoma multiforme tumor tissue. J Surg Oncol 1998; 67:234–241.
5. Jackson TR, Blair AC, Marshall J, Goedert M, Hanley MR: The *mas* oncogene encodes an angiotensin receptor. Nature 1988;335: 437–440.
6. Julius D, Livelli T, Jessell T, Axel R: Ectopic expression of the serotonin lc receptor and the triggering of malignant transformation. Science 1989;244:1057–1062.
7. Gutkind J, Novotny EA, Brann MR, Robbins KC: Muscarinic acetylcholine receptor subtypes as agonist–dependent oncogenes. Proc Natl Acad Sci USA 1991;88:4703–4707.
8. Allen LF, Lefkowitz RJ, Caron MG, Cotecchia S: G-protein-coupled receptor genes as protooncogenes: Constitutively activating mutation of the α_{1B}–adrenergic receptor enhances mitogenesis and tumorgenicity. Proc Natl Acad Sci USA 1991;88:11354–11358.
9. Nomura H, Nielsen B, Matsushima K: Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. Int Immunol 1993;5:1239–1249.
10. Federsppiel B, Melhado IG, Duncan AMV, et al.: Molecular cloning of the cDNA and chromosomal localization of the gene for a

putative seven–transmembrane segment (7–TMS) receptor isolated from human spleen. Genomics 1993;16:707–712.
11. Loetscher M, Geiser T, O'Reilly T, et al.: Cloning of a human seven–transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. J Biol Chem 1994;269:232–237.
12. Feng Y, Broder CC, Kennedy PE, Berger EA: HIV–1 entry cofactor: Functional cDNA cloning of a seven–transmembrane, G protein-coupled receptor. Science 1996;272:872–877.
13. Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner, JD: CD4–independent infection by HIV–2 is mediated by fusin/CXCR4. Cell 1996;87:745–756.
14. Riezman H, Woodman PG, Meer GV, Marsh M: Molecular mechanisms of endocytosis. Cell 1997;91:731–738.
15. D'Apuzzo M, Rolink A, Loetscher M, et al.: The chemokine SDF–1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. Eur J Immunol 1997;27:1788–1793.
16. Jazin EE, Soderstrom S, Ebendal T, Larhammar D: Embryonic expression of the mRNA for the rat homologue of the fusin/CXCR–4 HIV1 co-receptor. J Neuroimmunol 1997;79:148–154.
17. Sehgal A, Keener C, Boynton AL, et al.: Isolation and characterization of a novel gene from human glioblastoma multiforme tumor tissue. Int J Cancer 1997;71:565–572.
18. Sambrook J, Fritsch EF, Maniatis T: ''Molecular Cloning: A Laboratory Manual.'' 2nd Ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
19. Sehgal A, Ricks S, Keener C, et al.: Cloning, sequence, and developmental expression analysis of C4–2, a potential brain tumor-suppressor gene. J Surg Oncol 1997;65:249–257.
20. Hesselgesser J, Miller MH, DelVecchio V, et al.: CD4–independent association between HIV–1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. Curr Biol 1997;7:112–121.
21. Volin MV, Joseph L, Shockley MS, Davies P: Chemokine receptor CXCR–4 expression in endothelium. Biochem Biophys Res Commun 1998;242:46–53.
22. Sharif M, Sasakawa N, Michael RH: Malignant transformation by G protein coupled hormone receptor. [Review.] Mol Cell Endocrinol 1994;100:115–119.
23. Haribabu B, Richardson RM, Fisher I, et al.: Regulation of human chemokine receptors CXCR4. J Biol Chem 1997;272:28726–28731.